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Radiation sensitivity of *Salmonella* isolates relative to resistance to ampicillin, chloramphenicol or gentamicin ☆

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Abstract

Antibiotic resistance of inoculated bacteria is a commonly used selective marker. Bacteria resistant to the antibiotic nalidixic acid have been shown to have an increased sensitivity to irradiation. The purpose of this research was to screen a collection of *Salmonella* isolates for antibiotic resistance and determine the association, if any, of antibiotic resistance with radiation sensitivity. Twenty-four clinical isolates of *Salmonella* were screened for native resistance to multiple concentrations of ampicillin (Amp), chloramphenicol (Chl), or gentamicin (Gm). Test concentrations were chosen based on established clinical minimum inhibitory concentration (MIC) levels, and isolates were classified as either sensitive or resistant based on their ability to grow at or above the MIC. *Salmonella* cultures were grown overnight at (37 °C) in antibiotic-amended tryptic soy broth (TSB). Native resistance to Gm was observed with each of the 24 isolates (100%). Eight isolates (33%) were shown to be resistant to Amp, while seven isolates (29%) were shown to be resistant to Chl. In separate experiments, *Salmonella* cultures were grown overnight (37 °C) in TSB, centrifuged, and the cell pellets were re-suspended in phosphate buffer. The samples were then gamma irradiated at doses up to 1.0 kGy. The D_{10} values (the ionizing radiation dose required to reduce the viable number of microorganisms by 90%) were determined for the 24 isolates and they ranged from 0.181 to 0.359 kGy. No correlation was found between the D_{10} value of the isolate and its sensitivity or resistance to each of the three antibiotics. Resistance to Amp or Chl is suggested as appropriate resistance marker for *Salmonella* test strains to be used in studies of irradiation.

Keywords: Antibiotic resistance; Irradiation; Nalidixic acid; *Salmonella*; D_{10} value; Selective marker

1. Introduction

In the past two decades, the consumption of fresh fruits and vegetables in the US has increased, and the geographic sources and distribution of fresh produce have expanded greatly (Tauxe et al., 1997). This increase is due, in part, to consumer demand for more ready-to-eat foods that require little preparation, as well as a trend towards healthier foods. However, most produce receives minimal processing and can be a source of bacterial contamination. The demand for these fresh products has placed greater requirements on producers to ensure food safety (Blackburn and Davies, 1994). Public health officials have documented an increase in the number of produce-associated foodborne illnesses (Thayer and Rajkowski, 1999; Sivapalasingam et al.,

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2004). This increase has been attributed to new processing techniques and a more widespread distribution of the food supply. Foley et al. (2002) offered three means by which minimally processed fruits and vegetables may become contaminated: 1) minimal processing that does not provide sterility, 2) the presence of cut surfaces or damaged plant tissues that provide a portal of entry for microbial colonization, and 3) methods used to extend shelf-life that allow longer periods for microbial growth and proliferation. An alternative antimicrobial technology is the use of ionizing radiation (Niemira, 2003).

Complicating the situation further is the emergence of foodborne pathogens that are antibiotic resistant. Recent reports indicate a greater antibiotic resistance than ten or fifteen years ago, and that exposure of foodborne pathogens to antibiotics in production agriculture results in the emergence of antimicrobial resistant strains (Golding and Matthews, 2004). A wide range of antibiotics is used in crop production for disease control, and in animal agriculture for therapy, prophylaxis, and growth promotion (Golding and Matthews, 2004). Outbreaks of salmonellosis in humans have been attributed to consumption of contaminated tomatoes, mustard cress, bean sprouts, cantaloupe, watermelon, and onions (Beuchat, 1995; Horby et al., 2003; Sivapalasingam et al., 2004).

Studies are underway to find suitable methods to control human foodborne pathogens associated with fresh fruits, fruit juices, fresh cut vegetables or salads, sprouts and seeds (Thayer and Rajkowski, 1999). Due to the high background microflora observed on produce, studies using these products often use selective media to isolate the organisms of interest (Niemira, 2003). However, cells injured by the chosen antimicrobial treatment may not be able to grow on the selective media, leading to overestimates of the efficacy of the treatment (Ray, 1979). In order to avoid this, Blackburn and Davies (1994) developed antibiotic-resistant strains and antibiotic-amended media for the study of foodborne pathogens. Bacteria resistant to the antibiotic nalidixic acid (Nal^R) have been validated for use as a marker in studies of chemical interventions (Blackburn and Davies, 1994; Taormina and Beuchat, 1999). Until recently, it has not been validated for use in food irradiation studies. Niemira (2005a) demonstrated that the radiation sensitivity of three strains of *Escherichia coli* O157:H7 increased after being induced to be Nal^R. A similar increase in radiation sensitivity was subsequently observed in Nal^S isolates of *Salmonella* induced to become Nal^R (Niemira, 2005b). In these studies, the Nal^R daughter isolates were generated by unguided selection and reculturing in media with successively higher Nal concentrations. The specific molecular nature of the resistance mechanisms employed by these isolates was uncharacterized.

The mode of action of Nal is disruption of DNA replication. As ionizing radiation can cause damage to DNA and interfere with DNA repair, Niemira (2005a) hypothesized that the Nal^R bacteria may have altered nucleic acid synthesis and/or repair systems, which make them more susceptible to ionizing radiation. Antibiotics with modes of action unrelated to nucleic acid structure and function may be more appropriate selective agents for use in irradiation studies; but to our knowledge, this area has not been explored. Ampicillin (Amp) is an antibiotic which affects cell wall synthesis by blocking peptidoglycan synthesis. Chloramphenicol (Chl) blocks protein synthesis by binding to the bacterial 50S ribosomal subunit. Gentamicin (Gm) also blocks protein synthesis, but by binding to the 30S ribosomal subunit (Gebreyes et al., 2004; Golding and Matthews, 2004; NCCLS, 2003). The objectives of this research were to 1) screen 24 produce isolates for native resistance to Amp, Chl, and/or Gm, 2) determine the radiation D_{10} values for the 24 isolates, 3) compare the D_{10} values of the antibiotic-resistant vs. antibiotic-sensitive isolates for each antibiotic separately and 4) determine the correlation of antibiotic resistance with sensitivity to radiation.

2. Materials and methods

2.1. Microorganisms

Twenty-four clinical isolates of *Salmonella* from various sources (Table 1) were obtained from Dr. Ethan Solomon (USDA, ARS, ERRC, Wyndmoor, PA). They were propagated in tryptic soy broth (TSB, Difco, Detroit, MI) and maintained at 2 °C. Individual tubes of fresh TSB were inoculated before each experiment. They were grown for 16 h at 37 °C with agitation. The starting inoculum concentration was determined by serial dilution with sterile Butterfield's phosphate buffer (BPB, Applied Research Institute, Newtown, CT). The cell concentration in overnight TSB cultures (37 °C) was determined to be $\sim 10^8$ cfu/mL.

2.2. Radiation D_{10} determination

Salmonella strains were grown overnight (37 °C) in 10 mL tubes of TSB. The cultures were centrifuged (5000*g*) for 10 min to pelletize the cells. The pelletized cells were re-suspended in 10 mL aliquots of BPB. For each strain, six tubes, one for each irradiation dose, were prepared from the re-suspended cells. The tubes were held at 2 °C until irradiation. The inoculated BPB solutions were irradiated to doses of 0.0 (control), 0.2, 0.4, 0.6, 0.8 and 1.0 kGy. The samples were irradiated using a self-contained cesium-137 gamma radiation source (Lockheed-Georgia, Marietta, GA) containing

Table 1
Radiation and antibiotic resistance of *Salmonella* clinical isolates

Isolate	Source	D_{10}[a]	R^2	Amp[b]	Gm[c]	Chl[d]
S. Montevideo	Tomato	0.181	0.961	s	r	s
S. gaminara F2172	Orange juice	0.190	0.847	s	r	s
S. worthington TX31	Alfalfa seed	0.191	0.964	s	r	s
S. poona G91-1574	Cantaloupe	0.195	0.982	r	r	r
S. poona PTVS-1	Cantaloupe	0.198	0.885	s	r	s
S. mbandaka RV1DHE	Alfalfa seed	0.199	0.966	s	r	s
S. bredeney 3VIPHE	Alfalfa seed	0.201	0.945	s	r	s
S. newport 02-216	Cantaloupe	0.203	0.943	s	r	s
S. michigan	Cantaloupe	0.204	0.944	s	r	s
S. poona 348	Cantaloupe	0.213	0.977	r	r	s
S. muenchen HERV2C	Alfalfa seed	0.227	0.984	s	r	r
S. poona 953	Ovine meat	0.229	0.915	r	r	r
S. poona 418	Octopus	0.230	0.972	r	r	r
S. mbandaka 00916-1	Cantaloupe	0.234	0.981	r	r	s
S. st. paul FSIS-039	Beef	0.248	0.973	r	r	r
S. hidalgo 02-517-2	Cantaloupe	0.262	0.954	s	r	s
S. typhimurium DT104	Meat	0.270	0.921	r	r	r
S. st. paul 02-517-2	Cantaloupe	0.273	0.970	s	r	s
S. typhimurium RO45	Cantaloupe	0.332	0.971	s	r	s
S. baildon 61-99	Tomato	0.335	0.969	s	r	s
S. stanley H0558	Sprouts	0.339	0.976	s	r	s
S. enteritidis 15159	Orange juice	0.348	0.967	s	r	s
S. gaminara 02-615	Cantaloupe	0.352	0.873	r	r	r
S. anatum F4317	Sprouts	0.359	0.977	s	r	s

s, sensitivity to antibiotic; r, resistance to antibiotic.
[a]The D_{10} value is defined as the radiation dose required to produce 1–$\log_{10}$ or 90% reduction in viable microorganisms.
[b]Ampicillin.
[c]Gentamicin.
[d]Chloramphenicol.

23 ^{137}Cs pencils placed in annular array. The dose rate was 0.090 kGy/min. The dose rate was established using alanine transfer dosimeters from the National Institutes of Standards and Technology (Gaithersburg, MD). Samples were oriented with similar geometry at the center of the irradiation field for all treatments. During irradiation, the gas phase of liquid nitrogen was flushed into the sample chamber in order to maintain the temperature of 2 °C. Alanine pellets (Bruker Inc., Billarica, MA) were used for dosimetry. The pellets were read on a Bruker EMS 104 EPR analyzer and compared with a previously determined standard curve. Actual doses were within 5% of the nominal dose.

After irradiation, samples were immediately serially diluted with sterile BPB using 10-fold dilutions. After dilution, 1 mL samples were taken and pour-plated with tryptic soy agar (TSA, Difco, Detroit, MI). Three pour plates per dilution were prepared, inverted and incubated at 37 °C for 24 h. Plate colonies were counted with a Spiral Biotech laser bacteria colony counter (Exotech Inc., Gaithersburg, MD).

The data for each sample were normalized against the control and plotted as the $\log_{10}$ reduction using the nominal doses. Each experiment was performed three times, and the data pooled. The slopes of the individual survivor curves were calculated with linear regression (SigmaPlot 5.0, SPSS Inc., Chicago, IL), and compared among all of the isolates using analysis of covariance (ANCOVA, Excel 97, Microsoft Corp., Redmond, WA). The ionizing radiation D_{10} value was calculated by taking the negative reciprocal of the survivor curve slope (Quattro Pro, Corel Corp., Ottawa, Ont., Canada).

2.3. Screening for antibiotic resistance

The isolates were screened for resistance to Amp, Chl or Gm using test concentrations based on established clinical minimum inhibitory concentration (MIC) levels: 32 μg/mL for Amp and Chl, and 16 μg/mL for Gm (Gebreyes et al., 2004). For Amp and Chl, the test concentrations used were 0 (control), 10, 20, 30, 40, and

50 μg/mL. Based on the clinical MIC level, growth at 40 μg/mL or higher resulted in classification as resistant. For Gm, the test concentrations used were 0 (control), 5, 10, 15, 20, and 25 μg/mL. Growth at 20 μg/mL or higher resulted in classification as resistant. Separate freshly prepared tubes of sterile TSB were amended with each antibiotic to the specified concentrations using a 0.22 μm-filter-sterilized stock solution. Aliquots (200 μL) of the antibiotic-amended TSB were dispensed into 96 well plates (Nunc, Roskilde, Denmark) such that each column contained one well of each concentration. Fresh overnight cultures of each of the isolates were grown in TSB as described above, and used to inoculate the antibiotic-amended wells, 10 μL per well. The plates were read at 600 nm using a Opsys MR 96 microwell plate reader (Dynex Technologies, Chantilly, VA) to establish an absorbance baseline. The plates were covered and incubated at 37 °C overnight and absorbance taken again. For each antibiotic/isolate/concentration combination, the baseline absorbance was subtracted from the post-incubation absorbance to determine the change in turbidity. The analysis was repeated for each isolate using at least four separate plates. For a given antibiotic, the data were pooled for each isolate, and the absorbance at each concentration was compared to the 0 μg/mL control using a one-way analysis of variance test (ANOVA, Dunnet, SigmaStat, version 2.0, SPSS Chicago, IL), to identify the MIC. An isolate was identified as resistant or sensitive based on the MIC.

The collection of isolates was ranked by radiation D_{10} values and grouped into quartiles, with 6 isolates per quartile. To determine the degree of interrelationship between radiation D_{10} value and antibiotic resistance, a χ^2 test was performed (SigmaStat 5.0, SPSS Inc., Chicago, IL) on the distribution of resistant vs. sensitive isolates for each antibiotic, based on these quartile groupings.

3. Results

Ionizing radiation effectively reduced the viable population of each of the isolates (Fig. 1). The radiation sensitivity was significantly variable among the various isolates (Table 1). The minimum D_{10} value observed was 0.181 kGy, while the maximum D_{10} value was 0.359 kGy, almost double that of the minimum value. An isolate's source was not an indication of its D_{10} value. The D_{10} values are statistically similar to nearest neighbors, and form statistical clusters based on the ANCOVA results. To facilitate visualization of this clustering, the twenty-four *Salmonella* isolates were plotted with increasing D_{10} value (Fig. 2). Five major statistical clusters are observed in the data and are marked by ellipses in Fig. 2. The first cluster includes *S. montevideo*, *S. gaminara* F2172, *S. worthington* TX31, and *S. poona* G 91-1574. The second cluster includes *S. poona* G91-1574, *S. poona* PTVS-1, *S. mbandaka* RV1DHE, *S. bredeney* 3VIPHE, *S. newport* 02-216, *S. michigan,* and *S. poona* 348. The third cluster includes *S. poona* 348, *S. muenchen* HERV2C, *S. poona* 953, *S. poona* 418, *S. mbandaka* 00916-1, and *S. st. paul* FSIS-039. The fourth cluster includes the isolates *S. st. paul* FSIS-039, *S. hidalgo* 02-517-2, *S. typhimurium* DT104, and *S. st. paul* 02-517-2. The fifth and final cluster does not overlap with any of the previous groupings. It includes *S. typhimurium* RO45, *S. baildon* 61-99, *S. stanley* H0558, *S. enteritidis* 15159, *S. gaminara* 02-615, and *S. anatum* F4317. It should be noted that while the overlap between clusters is generally by nearest neighbor, isolates in the center of some clusters with larger standard errors have a broader range of statistical similarity. Examples of these are *S. poona* G91-1574, *S. mbandaka* RV1DHE, *S. poona* 953, and *S. typhimurium* DT104.

Resistant isolates were observed to grow at the highest levels of antibiotic tested. Of the 24 isolates tested, 8 (33%) were resistant to Amp, 7 (29%) were resistant to Chl, and all 24 (100%) were shown to be resistant to Gm (Table 1). Isolates resistant to Amp, but not to Chl, include *S. poona* 348 and *S. mbandaka* 00916-1. One isolate (*S. muenchen* HERV2C) is resistant to Chl, but not to Amp. Six isolates were resistant to all the three antibiotics. They include *S. poona* G91-1574, *S. poona* 953, *S. poona* 418, *S. st. paul* FSIS-039, *S. typhimurium* DT104, and *S. gaminara* 02-615.

χ^2 analysis shows that the resistant isolates are not significantly associated with any one quartile of radiation resistance. No correlation between antibiotic resistance and irradiation sensitivity was observed.

4. Discussion

The *Salmonella* isolates tested in this study had D_{10} values which ranged from 0.181 to 0.359 kGy. This range is consistent with previously published D_{10} values for *Salmonella* (Thayer and Rajkowski, 1999; Niemira, 2003). The statistical overlap and clustering of the D_{10} values is not apparently related to the foods from which the isolates were associated. The cluster with the highest D_{10} values contains isolates with a similar provenance as those found in the other clusters, such as isolates from cantaloupe, orange juice and tomato. A comprehensive understanding of why a given isolate may be more or less sensitive to irradiation than related isolates of the same pathogen has yet to be formulated (Thayer and Rajkowski, 1999; Niemira, 2003). It is likely that a survey of a larger number of isolates would result in a more linear progression of D_{10} values, with values that

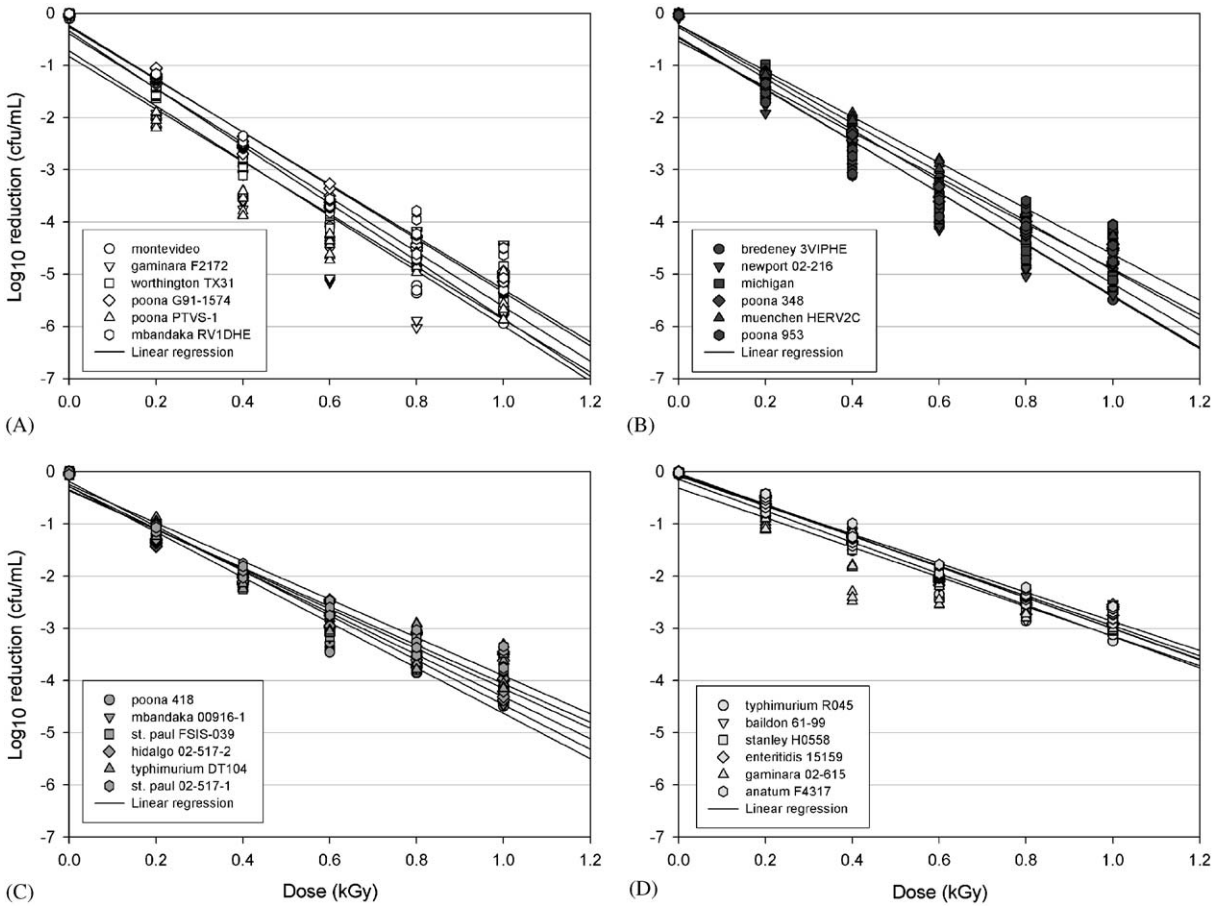

Fig. 1. Radiation sensitivity of twenty-four *Salmonella* clinical isolates in buffer. Isolates are divided in quartiles (A, B, C and D) by increasing radiation D_{10} values, six isolates per quartile.

bridge the gap seen among the statistical clusters observed among these 24 isolates.

Antibiotic resistance among human pathogens is of increasing concern, particularly with regard to the potential for emergence of multi-drug-resistant strains (Golding and Matthews, 2004). Each of the 24 isolates examined in this study were resistant to Gm. Published surveys indicate that the prevalence of resistance to Gm can depend on the collection being examined, and can vary from year to year. Of 87 *Salmonella* isolates collected from turkeys in 1998–2002, 44.8% were resistant to Gm, a proportion which varied from 21.4% to 61.1%, depending on the year (Malik et al., 2003). In a survey of 390 *Salmonella* abattoir isolates taken from hog, beef and chicken carcasses, approximately 5% were determined to be resistant to Gm (Larkin et al., 2004). A study of 29 *Salmonella* isolates taken from turkey production facilities and turkey intestinal tracts showed 52% of isolates resistant to Gm (Nayak et al., 2004). These studies cite the use of antibiotics in an agricultural setting as a likely factor in the emergence of single- or multi-drug-resistant pathogens. The 24 isolates in the present study were taken from a variety of sources, including produce, meat and juice products. A point of commonality among them is that each of these isolates was associated with a foodborne illness outbreak. For the purposes of the present study, the uniformity of resistance to Gm means that no conclusions may be drawn with regard to radiation sensitivity.

The majority of the isolates which were resistant to Amp were also resistant to Chl, suggesting the possibility of a co-inheritance of resistance genes to these antibiotics, possibly on a single plasmid. Confirmation of this possibility would further investigation and characterization of the molecular basis for the antibiotic resistance exhibited by these isolates. The results presented herein lead to the conclusion that there is no relationship between radiation D_{10} value, and antibiotic resistance to Amp and Chl (Table 2). Antibiotic-resistant bacteria were not preferentially associated with either low- or high-D_{10} value isolates

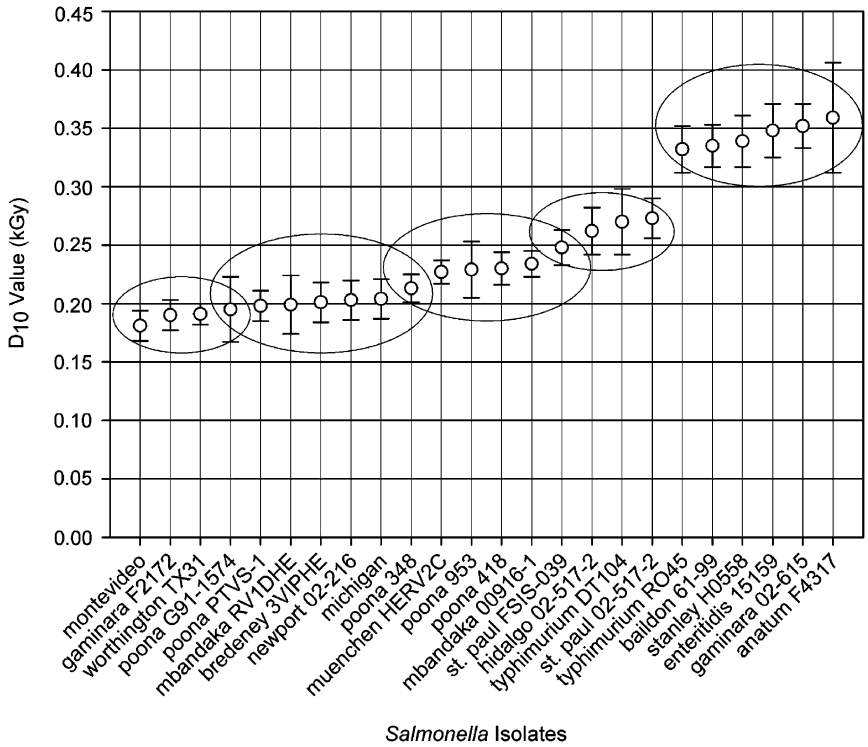

Fig. 2. *Salmonella* isolates in order of increasing radiation D_{10} value including error bars. Statistical clusters are marked by ellipses on the figure.

Table 2
χ^2 results showing no correlation between D_{10} value and antibiotic resistance

Quartile	Ampicillin		Gentamicin		Chloramphenicol	
	r	s	r	s	r	s
1st	1	5	6	0	1	5
2nd	2	4	6	0	2	4
3rd	4	2	6	0	3	3
4th	1	5	6	0	1	5
χ^2	$p = 0.212$		$p = 0.99$		$p = 0.528$	

s, sensitivity to antibiotic; r, resistance to antibiotic.

for either Amp or Chl. The modes of action of Amp (cell wall synthesis) and Chl (protein synthesis) are not related to nucleic acid structure or function. The primary mode of action of ionizing radiation is via hydrogen and hydroxyl radical molecules resulting from the ionization of water molecules within the target organism. These radicals can disrupt membranes and interfere with the functioning of proteins, but the most significant target within the cell is DNA, where radicals are responsible for strand breakage (Niemira, 2003). The quinolone antibiotic Nal inhibits DNA synthesis in bacteria by interfering with the functioning of DNA gyrase and topoisomerase IV (Georgopapadakou et al., 1987; Khadursky and Cozzarelli, 1998). Nal^R strains of *E. coli* O157:H7 and *Salmonella* have been recently shown to be more sensitive to irradiation than the Nal^S parent strains from which they were derived (Niemira, 2005a,b). In the *E. coli* O157:H7 study (Niemira, 2005a), Nal^R strains were also exposed to the antibiotic before, during and after irradiation with no change in survival or radiation sensitivity. The mechanism by which the induced Nal^R protects the cell from the antibiotic was therefore determined to be insensitive to the irradiation process. The Nal^R isolates used in that study were

generated using unguided selection rather than by a transformation with a specific insertion of a DNA construct. The precise molecular nature of the Nal resistance in those isolates was therefore not characterized. Nevertheless, it was concluded that the significant differences in D_{10} values between Nal^R isolates and their Nal^S parent isolates would lead to an overestimate of the efficacy of ionizing radiation against *E. coli* 0157:H7 in studies using the Nal^R daughter isolates (Niemira, 2005a).

In the present study, resistance to Amp or Chl was not associated with sensitivity to ionizing radiation. Therefore, bacterial resistance to Amp^R or Chl^R, rather than Nal^R, are suggested as more appropriate markers in irradiation studies of inoculated food products using *Salmonella*.

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